

**PROTEINS HAVING SERINE/THREONINE KINASE DOMAINS,  
CORRESPONDING NUCLEIC ACID MOLECULES, AND THEIR USE**

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PROTEINS HAVING SERINE/THREONINE KINASE DOMAINS,  
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Field of the Invention

This invention relates to proteins having serine/threonine kinase domains, corresponding nucleic acid molecules, and their use.

Background of the Invention

The transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily consists of a family of structurally-related proteins, including three different mammalian isoforms of TGF- $\beta$  (TGF- $\beta$ 1,  $\beta$ 2 and  $\beta$ 3), activins, inhibins, müllerian-inhibiting substance and bone morphogenic proteins (BMPs) (for reviews see Roberts and Sporn, (1990) Peptide Growth Factors and Their Receptors, Pt.1, Sporn and Roberts, eds. (Berlin: Springer - Verlag) pp 419-472; Moses et al (1990) Cell 63, 245-247). The proteins of the TGF- $\beta$  superfamily have a wide variety of biological activities. TGF- $\beta$  acts as a growth inhibitor for many cell types and appears to play a central role in the regulation of embryonic development, tissue regeneration, immuno-regulation, as well as in fibrosis and carcinogenesis (Roberts and Sporn (199) see above).

Activins and inhibins were originally identified as factors which regulate secretion of follicle-stimulating hormone secretion (Vale et al (1990) Peptide Growth Factors and Their Receptors, Pt.2, Sporn and Roberts, eds. (Berlin: Springer-Verlag) pp.211-248). Activins were also shown to induce the differentiation of haematopoietic progenitor cells (Murata et al (1988) Proc. Natl. Acad. Sci. USA 85, 2434 - 2438; Eto et al (1987) Biochem. Biophys. Res. Commun. 142, 1095-1103) and induce mesoderm formation in *Xenopus* embryos (Smith et al (1990) Nature 345, 729-731; van den Eijnden-Van Raaij et al (1990) Nature 345, 732-734).

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BMPs or osteogenic proteins which induce the formation of bone and cartilage when implanted subcutaneously (Wozney et al (1988) Science 242, 1528-1534), facilitate neuronal differentiation (Paralkar et al (1992) J. Cell Biol. 119, 1721-1728) and induce monocyte chemotaxis (Cunningham et al (1992) Proc. Natl. Acad. Sci. USA 89, 11740-11744). Müllerian-inhibiting substance induces regression of the Müllerian duct in the male reproductive system (Cate et al (1986) Cell 45, 685-698), and a glial cell line-derived neurotrophic factor enhances survival of midbrain dopaminergic neurons (Lin et al (1993) Science 260, 1130-1132). The action of these growth factors is mediated through binding to specific cell surface receptors.

Within this family, TGF- $\beta$  receptors have been most thoroughly characterized. By covalently cross-linking radio-labelled TGF- $\beta$  to cell surface molecules followed by polyacrylamide gel electrophoresis of the affinity-labelled complexes, three distinct size classes of cell surface proteins (in most cases) have been identified, denoted receptor type I (53 kd), type II (75 kd), type III or betaglycan (a 300 kd proteoglycan with a 120 kd core protein) (for a review see Massague (1992) Cell 69 1067-1070) and more recently endoglin (a homodimer of two 95 kd subunits) (Cheifetz et al (1992) J. Biol. Chem. 267 19027-19030). Current evidence suggests that type I and type II receptors are directly involved in receptor signal transduction (Segarini et al (1989) Mol. Endo., 3, 261-272; Laiho et al (1991) J. Biol. Chem. 266, 9100-9112) and may form a heteromeric complex; the type II receptor is needed for the binding of TGF- $\beta$  to the type I receptor and the type I receptor is needed for the signal transduction induced by the type II receptor (Wrana et al (1992) Cell, 71, 1003-1004). The type III receptor and endoglin may have more indirect roles, possibly by facilitating the binding of ligand to type II receptors (Wang et al (1991) Cell, 67 797-805; López-Casillas et al (1993) Cell, 73 1435-1444).

Binding analyses with activin A and BMP4 have led to the identification of two co-existing cross-linked affinity complexes of 50-60 kDa and 70-80 kDa on responsive cells (Hino et al (1989) J. Biol. Chem. 264, 10309 - 10314; Mathews and Vale (1991), Cell 68, 775-785; Paralker et al (1991) Proc. Natl. Acad. Sci. USA 87, 8913-8917). By analogy with TGF- $\beta$  receptors they are thought to be signalling receptors and have been named type I and type II receptors.

Among the type II receptors for the TGF- $\beta$  superfamily of proteins, the cDNA for the activin type II receptor (ActRII) was the first to be cloned (Mathews and Vale (1991) Cell 65, 973-982). The predicted structure of the receptor was shown to be a transmembrane protein with an intracellular serine/threonine kinase domain. The activin receptor is related to the C. elegans daf-1 gene product, but the ligand is currently unknown (Georgi et al (1990) Cell 61, 635-645). Thereafter, another form of the activin type II receptor (activin type IIB receptor), of which there are different splicing variants (Mathews et al (1992), Science 225, 1702-1705; Attisano et al (1992) Cell 68, 97-108), and the TGF- $\beta$  type II receptor (T $\beta$ RII) (Lin et al (1992) Cell 68, 775-785) were cloned, both of which have putative serine/threonine kinase domains.

#### Summary of the Invention

The present invention involves the discovery of related novel peptides, including peptides having the activity of those defined herein as SEQ ID Nos. 2, 4, 8, 10, 12, 14, 16 and 18. Their discovery is based on the realisation that receptor serine/threonine kinases form a new receptor family, which may include the type II receptors for other proteins in the TGF- $\beta$  superfamily. To ascertain whether there were other members of this family of receptors, a protocol was designed to clone ActRII/daf I related cDNAs. This approach made use of the polymerase chain reaction (PCR), using degenerate primers based upon the amino-acid sequence similarity between kinase domains

of the mouse activin type II receptor and daf-I gene products.

This strategy resulted in the isolation of a new family of receptor kinases called Activin receptor like  
 5 kinases (ALK's) 1-6. These cDNAs showed an overall 33-39% sequence similarity with ActRII and TGF- $\beta$  type II receptor and 40-92% sequence similarity towards each other in the kinase domains.

Soluble receptors according to the invention comprise  
 10 at least predominantly the extracellular domain. These can be selected from the information provided herein, prepared in conventional manner, and used in any manner associated with the invention.

Antibodies to the peptides described herein may be  
 15 raised in conventional manner. By selecting unique sequences of the peptides, antibodies having desired specificity can be obtained.

The antibodies may be monoclonal, prepared in known manner. In particular, monoclonal antibodies to the  
 20 extracellular domain are of potential value in therapy.

Products of the invention are useful in diagnostic methods, e.g. to determine the presence in a sample for an analyte binding therewith, such as in an antagonist assay. Conventional techniques, e.g. an enzyme-linked  
 25 immunosorbent assay, may be used.

Products of the invention having a specific receptor activity can be used in therapy, e.g. to modulate conditions associated with activin or TGF- $\beta$  activity. Such conditions include fibrosis, e.g. liver cirrhosis and  
 30 pulmonary fibrosis, cancer, rheumatoid arthritis and glomeronephritis.

#### Brief Description of the Drawings

*Sub G1* Figure 1 shows the alignment of the serine/threonine (S/T) kinase domains (I-VIII) of related receptors from  
 35 transmembrane proteins, including embodiments of the present invention. The nomenclature of the subdomains is accordingly to Hanks et al (1988). *B2 B2*

Figures 2A to 2D shows the sequences and characteristics of the respective primers used in the initial PCR reactions. The nucleic acid sequences are also given as SEQ ID Nos. 19 to 22.

5 Figure 3 is a comparison of the amino-acid sequences  
 B of human activin type II receptor (Act R-II), mouse activin  
 B type IIB receptor (Act R-IIB), human TGF- $\beta$  type II receptor  
 B (T $\beta$ R-II), human TGF- $\beta$  type I receptor (ALK-5), human  
 B activin receptor type IA (ALK-2), and type IB (ALK-4), ALKs  
 10 1 & 3, and mouse ALK-6. <sup>SEQ ID No: 34</sup>  
<sup>SEQ ID No: 35</sup>  
<sup>SEQ ID No: 36</sup>  
<sup>SEQ ID No: 10</sup>  
<sup>SEQ ID No: 4</sup>  
<sup>SEQ ID No: 8</sup>  
<sup>SEQ ID No: 2 and SEQ ID No: 6</sup>  
<sup>SEQ ID No: 18</sup>

Figure 4 shows, schematically, the structures for Daf-1, Act R-II, Act R-IIB, T $\beta$ R-II, T $\beta$ R-I/ALK-5, ALK's -1, -2 (Act RIA), -3, -4 (Act RIB) & -6.

Figure 5 shows the sequence alignment of the cysteine-rich domains of the ALKs T $\beta$ R-II, Act R-II, Act R-IIB and daf-1 receptors. <sup>INS B3</sup> <sup>INS B5</sup> <sup>INS B4</sup> <sup>INS B7</sup> <sup>INS B6</sup>

Figure 6 is a comparison of kinase domains of serine/threonine kinases, showing the percentage amino-acid identity of the kinase domains.

20 Figure 7 shows the pairwise alignment relationship between the kinase domains of the receptor serine/threonine kinases. The dendrogram was generated using the Jotun-Hein alignment program (Hein (1990) Meth. Enzymol. 183, 626-645).

25 Figure 8 depicts the phosphorylation of Smad-5 following interaction with ALK-1 but not following interaction with ALK-5.

#### Brief Description of the Sequence Listings

Sequences 1 and 2 are the nucleotide and deduced amino-acid sequences of cDNA for hALK-1 (clone HP57).

Sequences 3 and 4 are the nucleotide and deduced amino-acid sequences of cDNA for hALK-2 (clone HP53).

Sequences 5 and 6 are the nucleotide and deduced amino-acid sequences of cDNA for hALK-3 (clone ONF5).

Sequences 7 and 8 the nucleotide and deduced amino-acid sequences of cDNA for hALK-4 (clone 11H8), complemented with PCR product encoding extracellular domain.

5 Sequences 9 and 10 are the nucleotide and deduced amino-acid sequences of cDNA for hALK-5 (clone EMBLA).

Sequences 11 and 12 are the nucleotide and deduced amino-acid sequences of cDNA for mALK-1 (clone AM6).

10 Sequences 13 and 14 are the nucleotide and deduced amino-acid sequences of cDNA for mALK-3 (clones ME-7 and ME-D).

Sequences 15 and 16 are the nucleotide and deduced amino-acid sequences of cDNA for mALK-4 (clone 8a1).

15 Sequences 17 and 18 are the nucleotide and deduced amino-acid sequences of cDNA for mALK-6 (clone ME-6).

Sequence 19 (B1-S) is a sense primer, extracellular domain, cysteine-rich region, BamHI site at 5' end, 28-mer, 64-fold degeneracy.

20 Sequence 20 (B3-S) is a sense primer, kinase domain II, BamHI site at 5' end, 25-mer, 162-fold degeneracy.

Sequence 21 (B7-S) is a sense primer, kinase domain VIB, S/T kinase specific residues, BamHI site at 5' end, 24-mer, 288-fold degeneracy.

25 Sequence 22 (E8-AS) is an anti-sense primer, kinase domain, S/T kinase-specific residues EcoRI site at 5' end, 20-mer, 18-fold degeneracy.

Sequence 23 is an oligonucleotide probe.

Sequence 24 is a 5' primer.

Sequence 25 is a 3' primer.

30 Sequence 26 is a consensus sequence in Subdomain I.

Sequences 27 and 28 are novel sequence motifs in Subdomain VIB.

Sequence 29 is a novel sequence motif in Subdomain VIII.

Description of the Invention

As described in more detail below, nucleic acid sequences have been isolated, coding for a new sub-family of serine/threonine receptor kinases. The term nucleic acid molecules as used herein refers to any sequence which codes for the murine, human or mammalian form, amino-acid sequences of which are presented herein. It is understood that the well known phenomenon of codon degeneracy provides for a great deal of sequence variation and all such varieties are included within the scope of this invention.

The nucleic acid sequences described herein may be used to clone the respective genomic DNA sequences in order to study the genes' structure and regulation. The murine and human cDNA or genomic sequences can also be used to isolate the homologous genes from other mammalian species. The mammalian DNA sequences can be used to study the receptors' functions in various in vitro and in vivo model systems.

As exemplified below for ALK-5 cDNA, it is also recognised that, given the sequence information provided herein, the artisan could easily combine the molecules with a pertinent promoter in a vector, so as to produce a cloning vehicle for expression of the molecule. The promoter and coding molecule must be operably linked via any of the well-recognized and easily-practised methodologies for so doing. The resulting vectors, as well as the isolated nucleic acid molecules themselves, may be used to transform prokaryotic cells (e.g. E. coli), or transfect eukaryotes such as yeast (S. cerevisiae), PAE, COS or CHO cell lines. Other appropriate expression systems will also be apparent to the skilled artisan.

Several methods may be used to isolate the ligands for the ALKs. As shown for ALK-5 cDNA, cDNA clones encoding the active open reading frames can be subcloned into expression vectors and transfected into eukaryotic cells, for example COS cells. The transfected cells which can express the receptor can be subjected to binding assays for



radioactively-labelled members of the TGF- $\beta$  superfamily (TGF- $\beta$ , activins, inhibins, bone morphogenic proteins and müllerian-inhibiting substances), as it may be expected that the receptors will bind members of the TGF- $\beta$  superfamily. Various biochemical or cell-based assays can be designed to identify the ligands, in tissue extracts or conditioned media, for receptors in which a ligand is not known. Antibodies raised to the receptors may also be used to identify the ligands, using the immunoprecipitation of the cross-linked complexes. Alternatively, purified receptor could be used to isolate the ligands using an affinity-based approach. The determination of the expression patterns of the receptors may also aid in the isolation of the ligand. These studies may be carried out using ALK DNA or RNA sequences as probes to perform in situ hybridisation studies.

The use of various model systems or structural studies should enable the rational development of specific agonists and antagonists useful in regulating receptor function. It may be envisaged that these can be peptides, mutated ligands, antibodies or other molecules able to interact with the receptors.

The foregoing provides examples of the invention Applicants intend to claim which includes, inter alia, isolated nucleic acid molecules coding for activin receptor-like kinases (ALKs), as defined herein. These include such sequences isolated from mammalian species such as mouse, human, rat, rabbit and monkey.

The following description relates to specific embodiments. It will be understood that the specification and examples are illustrative but not limitative of the present invention and that other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

Preparation of mRNA and Construction of a cDNA Library

For construction of a cDNA library, poly (A)<sup>+</sup> RNA was isolated from a human erythroleukemia cell line (HEL 92.1.7) obtained from the American Type Culture Collection (ATCC TIB 180). These cells were chosen as they have been shown to respond to both activin and TGF- $\beta$ . Moreover leukaemic cells have proved to be rich sources for the cloning of novel receptor tyrosine kinases (Partanen et al (1990) Proc. Natl. Acad. Sci. USA 87, 8913-8917 and (1992) Mol. Cell. Biol. 12, 1698-1707). (Total) RNA was prepared by the guanidinium isothiocyanate method (Chirgwin et al (1979) Biochemistry 18, 5294-5299). mRNA was selected using the poly-A or poly AT tract mRNA isolation kit (Promega, Madison, Wisconsin, U.S.A.) as described by the manufacturers, or purified through an oligo (dT)-cellulose column as described by Aviv and Leder (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412. The isolated mRNA was used for the synthesis of random primed (Amersham) cDNA, that was used to make a  $\lambda$ gt10 library with  $1 \times 10^5$  independent cDNA clones using the Riboclone cDNA synthesis system (Promega) and  $\lambda$ gt10 in vitro packaging kit (Amersham) according to the manufacturers' procedures. An amplified oligo (dT) primed human placenta  $\lambda$ ZAPII cDNA library of  $5 \times 10^5$  independent clones was used. Poly (A)<sup>+</sup> RNA isolated from AG1518 human foreskin fibroblasts was used to prepare a primary random primed  $\lambda$ ZAPII cDNA library of  $1.5 \times 10^6$  independent clones using the RiboClone cDNA synthesis system and Gigapack Gold II packaging extract (Stratagene). In addition, a primary oligo (dT) primed human foreskin fibroblast  $\lambda$ gt10 cDNA library (Claesson-Welsh et al (1989) Proc. Natl. Acad. Sci. USA. 86 4917-4912) was prepared. An amplified oligo (dT) primed HEL cell  $\lambda$ gt11 cDNA library of  $1.5 \times 10^6$  independent clones (Poncz et al (1987) Blood 69 219-223) was used. A twelve-day mouse embryo  $\lambda$ EXIox cDNA library was obtained from Novagen (Madison, Wisconsin, U.S.A.); a mouse placenta  $\lambda$ ZAPII cDNA library was also used.

### Generation of cDNA Probes by PCR

For the generation of cDNA probes by PCR (Lee et al (1988) Science 239, 1288-1291) degenerate PCR primers were constructed based upon the amino-acid sequence similarity between the mouse activin type II receptor (Mathews and Vale (1991) Cell 65, 973-982) and daf-1 (George et al (1990) Cell 61, 635-645) in the kinase domains II and VIII. Figure 1 shows the aligned serine/threonine kinase domains (I-VIII), of four related receptors of the TGF- $\beta$  superfamily, i.e. hT $\beta$ R-II, mActR-IIB, mActR-II and the daf-1 gene product, using the nomenclature of the subdomains according to Hanks et al (1988) Science 241, 45-52.

Several considerations were applied in the design of the PCR primers. The sequences were taken from regions of homology between the activin type II receptor and the daf-1 gene product, with particular emphasis on residues that confer serine/threonine specificity (see Table 2) and on residues that are shared by transmembrane kinase proteins and not by cytoplasmic kinases. The primers were designed so that each primer of a PCR set had an approximately similar GC composition, and so that self complementarity and complementarity between the 3' ends of the primer sets were avoided. Degeneracy of the primers was kept as low as possible, in particular avoiding serine, leucine and arginine residues (6 possible codons), and human codon preference was applied. Degeneracy was particularly avoided at the 3' end as, unlike the 5' end, where mismatches are tolerated, mismatches at the 3' end dramatically reduce the efficiency of PCR.

In order to facilitate directional subcloning, restriction enzyme sites were included at the 5' end of the primers, with a GC clamp, which permits efficient restriction enzyme digestion. The primers utilised are shown in Figure 2. Oligonucleotides were synthesized using Gene assembler plus (Pharmacia - LKB) according to the manufacturers instructions.

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The mRNA prepared from HEL cells as described above was reverse-transcribed into cDNA in the presence of 50 mM Tris-HCl, pH 8.3, 8 mM MgCl<sub>2</sub>, 30 mM KCl, 10 mM dithiothreitol, 2mM nucleotide triphosphates, excess oligo (dT) primers and 34 units of AMV reverse transcriptase at 42°C for 2 hours in 40 µl of reaction volume. Amplification by PCR was carried out with a 7.5% aliquot (3 µl) of the reverse-transcribed mRNA, in the presence of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 M MgCl<sub>2</sub>, 0.01% gelatin, 0.2 mM nucleotide triphosphates, 1 µM of both sense and antisense primers and 2.5 units of Taq polymerase (Perkin Elmer Cetus) in 100 µl reaction volume. Amplifications were performed on a thermal cycler (Perkin Elmer Cetus) using the following program: first 5 thermal cycles with denaturation for 1 minute at 94°C, annealing for 1 minute at 50°C, a 2 minute ramp to 55°C and elongation for 1 minute at 72°C, followed by 20 cycles of 1 minute at 94°C, 30 seconds at 55°C and 1 minute at 72°C. A second round of PCR was performed with 3 µl of the first reaction as a template. This involved 25 thermal cycles, each composed of 94°C (1 min), 55°C (0.5 min), 72°C (1 min).

General procedures such as purification of nucleic acids, restriction enzyme digestion, gel electrophoresis, transfer of nucleic acid to solid supports and subcloning were performed essentially according to established procedures as described by Sambrook et al, (1989), Molecular cloning: A Laboratory Manual, 2<sup>nd</sup> Ed. Cold Spring Harbor Laboratory (Cold Spring Harbor, New York, USA).

Samples of the PCR products were digested with BamHI and EcoRI and subsequently fractionated by low melting point agarose gel electrophoresis. Bands corresponding to the approximate expected sizes, (see Table 1: ~460 bp for primer pair B3-S and E8-AS and ~ 140 bp for primer pair B7-S and E8-AS) were excised from the gel and the DNA was purified. Subsequently, these fragments were ligated into pUC19 (Yanisch-Perron et al (1985) Gene 33, 103-119), which

had been previously linearised with BamHI and EcoR1 and transformed into E. coli strain DH5 $\alpha$  using standard protocols (Sambrook et al, supra). Individual clones were sequenced using standard double-stranded sequencing techniques and the dideoxynucleotide chain termination method as described by Sanger et al (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467, and T7 DNA polymerase.

Employing Reverse Transcriptase PCR on HEL mRNA with the primer pair B3-S and E8-AS, three PCR products were obtained, termed 11.1, 11.2 and 11.3, that corresponded to novel genes. Using the primer pair B7-S and E8-AS, an additional novel PCR product was obtained termed 5.2.

TABLE 1

NAME OF PCR PRODUCT	PRIMERS	INSERT SIZE (bp)	SIZE OF DNA FRAGMENT IN mActRII/hTSRII CLONES (bp)	SEQUENCE IDENTITY WITH SEQUENCE mActRII/hTSRII (%)	SEQUENCE IDENTITY BETWEEN mActRII and TSRII (%)
11.1	B3-S/E8-AS	460	460	46/40	42
11.2	B3-S/E8-AS	460	460	49/44	47
11.3	B3-S/E8-AS	460	460	44/36	48
11.29	B3-S/E8-AS	460	460	ND/100	ND
9.2	B1-S/E8-AS	800	795	100/ND	ND
5.2	B7-S/E8-AS	140	143	40/38	60

### Isolation of cDNA Clones

The PCR products obtained were used to screen various cDNA libraries described supra. Labelling of the inserts of PCR products was performed using random priming method (Feinberg and Vogelstein (1983) Anal. Biochem, 132 6-13) using the Megaprime DNA labelling system (Amersham). The

oligonucleotide derived from the sequence of the PCR product 5.2 was labelled by phosphorylation with T4 polynucleotide kinase following standard protocols (Sambrook et al, supra). Hybridization and purification of positive bacteriophages were performed using standard molecular biological techniques.

The double-stranded DNA clones were all sequenced using the dideoxynucleotide chain-termination method as described by Sanger et al, supra, using T7 DNA polymerase (Pharmacia - LKB) or Sequenase (U.S. Biochemical Corporation, Cleveland, Ohio, U.S.A.). Compressions of nucleotides were resolved using 7-deaza-GTP (U.S. Biochemical Corp.) DNA sequences were analyzed using the DNA STAR computer program (DNA STAR Ltd. U.K.). Analyses of the sequences obtained revealed the existence of six distinct putative receptor serine/threonine kinases which have been named ALK 1-6.

To clone cDNA for ALK-1 the oligo (dT) primed human placenta cDNA library was screened with a radiolabelled insert derived from the PCR product 11.3; based upon their restriction enzyme digestion patterns, three different types of clones with approximate insert sizes. of 1.7 kb, 2 kb & 3.5 kb were identified. The 2 kb clone, named HP57, was chosen as representative of this class and subjected to complete sequencing. Sequence analysis of ALK-1 revealed a sequence of 1984 nucleotides including a poly-A tail (SEQ ID No. 1). The longest open reading frame encodes a protein of 503 amino-acids, with high sequence similarity to receptor serine/threonine kinases (see below). The first methionine codon, the putative translation start site, is at nucleotide 283-285 and is preceded by an in-frame stop codon. This first ATG is in a more favourable context for translation initiation (Kozak (1987) Nucl. Acids Res., 15, 8125-8148) than the second and third in-frame ATG at nucleotides 316-318 and 325-327. The putative initiation codon is preceded by a 5' untranslated sequence of 282 nucleotides that is GC-rich (80% GC), which

is not uncommon for growth factor receptors (Kozak (1991) J. Cell Biol., 115, 887-903). The 3' untranslated sequence comprises 193 nucleotides and ends with a poly-A tail. No bona fide poly-A addition signal is found, but there is a  
 5 sequence (AATACA), 17-22 nucleotides upstream of the poly-A tail, which may serve as a poly-A addition signal.

ALK-2 cDNA was cloned by screening an amplified oligo (dT) primed human placenta cDNA library with a radiolabelled insert derived from the PCR product 11.2.  
 10 Two clones, termed HP53 and HP64, with insert sizes of 2.7 kb and 2.4 kb respectively, were identified and their sequences were determined. No sequence difference in the overlapping clones was found, suggesting they are both derived from transcripts of the same gene.

15 Sequence analysis of cDNA clone HP53 (SEQ ID No. 3) revealed a sequence of 2719 nucleotides with a poly-A tail. The longest open reading frame encodes a protein of 509 amino-acids. <sup>SEQ ID No. 4</sup> The first ATG at nucleotides 104-106 agrees favourably with Kozak's consensus sequence with an A at  
 20 position 3. This ATG is preceded in-frame by a stop codon. There are four ATG codons in close proximity further downstream, which agree with the Kozak's consensus sequence (Kozak, supra), but according to Kozak's scanning model the first ATG is predicted to be the translation start site.  
 25 The 5' untranslated sequence is 103 nucleotides. The 3' untranslated sequence of 1089 nucleotides contains a polyadenylation signal located 9-14 nucleotides upstream from the poly-A tail. The cDNA clone HP64 lacks 498 nucleotides from the 5' end compared to HP53, but the  
 30 sequence extended at the 3' end with 190 nucleotides and poly-A tail is absent. This suggests that different polyadenylation sites occur for ALK-2. In Northern blots, however, only one transcript was detected (see below).

The cDNA for human ALK-3 was cloned by initially  
 35 screening an oligo (dT) primed human foreskin fibroblast cDNA library with an oligonucleotide (SEQ ID No. 23) derived from the PCR product 5.2. One positive cDNA clone

with an insert size of 3 kb, termed ON11, was identified. However, upon partial sequencing, it appeared that this clone was incomplete; it encodes only part of the kinase domain and lacks the extracellular domain. The most 5' sequence of ON11, a 540 nucleotide XbaI restriction fragment encoding a truncated kinase domain, was subsequently used to probe a random primed fibroblast cDNA library from which one cDNA clone with an insert size of 3 kb, termed ONF5, was isolated (SEQ ID No. 5). Sequence analysis of ONF5 revealed a sequence of 2932 nucleotides without a poly-A tail, suggesting that this clone was derived by internal priming. The longest open reading frame codes for a protein of 532 amino-acids. <sup>SEQ ID No. 6</sup> The first ATG codon which is compatible with Kozak's consensus sequence (Kozak, supra), is at 310-312 nucleotides and is preceded by an in-frame stop codon. The 5' and 3' untranslated sequences are 309 and 1027 nucleotides long, respectively.

ALK-4 cDNA was identified by screening a human oligo (dT) primed human erythroleukemia cDNA library with the radiolabelled insert of the PCR product 11.1 as a probe. One cDNA clone, termed 11H8, was identified with an insert size of 2 kb (SEQ ID No. 7). An open reading frame was found encoding a protein sequence of 383 amino-acids. <sup>SEQ ID No. 8</sup> encoding a truncated extracellular domain with high similarity to receptor serine/threonine kinases. The 3' untranslated sequence is 818 nucleotides and does not contain a poly-A tail, suggesting that the cDNA was internally primed. cDNA encoding the complete extracellular domain (nucleotides 1-366) was obtained from HEL cells by RT-PCR with 5' primer (SEQ ID No. 24) derived in part from sequence at translation start site of SKR-2 (a cDNA sequence deposited in GenBank data base, accession number L10125, that is identical in part to ALK-4) and 3' primer (SEQ ID No. 25) derived from 11H8 cDNA clone.



ALK-5 was identified by screening the random primed HEL cell  $\lambda$ gt 10 cDNA library with the PCR product 11.1 as a probe. This yielded one positive clone termed EMBLA (insert size of 5.3 kb with 2 internal EcoRI sites).

5 Nucleotide sequencing revealed an open reading frame of 1509 bp, coding for 503 amino-acids. The open reading frame was flanked by a 5' untranslated sequence of 76 bp, and a 3' untranslated sequence of 3.7 kb which was not completely sequenced. The nucleotide and deduced amino-

10 acid sequences of ALK-5 are shown in SEQ ID NOS. 9 and 10. <sup>(Nucleotide sequence)</sup> <sup>amino acid sequence</sup>

In the 5' part of the open reading frame, only one ATG codon was found; this codon fulfils the rules of translation initiation (Kozak, supra). An in-frame stop codon was found at nucleotides (-54)-(-52) in the 5' untranslated region. The predicted ATG start codon is followed by a stretch of hydrophobic amino-acid residues which has characteristics of a cleavable signal sequence. Therefore, the first ATG codon is likely to be used as a translation initiation site. A preferred cleavage site for the signal peptidase, according to von Heijne (1986) Nucl. Acid. Res. 14, 4683-4690, is located between amino-acid residues 24 and 25. The calculated molecular mass of the primary translated product of the ALK-5 without signal sequence is 53,646 Da.

25 Screening of the mouse embryo  $\lambda$ EX Iox cDNA library using PCR, product 11.1 as a probe yielded 20 positive clones. DNAs from the positive clones obtained from this library were digested with EcoRI and HindIII, electrophoretically separated on a 1.3% agarose gel and transferred to nitrocellulose filters according to established procedures as described by Sambrook et al, supra. The filters were then hybridized with specific probes for human ALK-1 (nucleotide 288-670), ALK-2 (nucleotide 1-581), ALK-3 (nucleotide 79-824) or ALK-4 (nucleotide 1178-1967). Such analyses revealed that a clone termed ME-7 hybridised with the human ALK-3 probe. However, nucleotide sequencing revealed that this clone was

incomplete, and lacked the 5' part of the translated region. Screening the same cDNA library with a probe corresponding to the extracellular domain of human ALK-3 (nucleotides 79-824) revealed the clone ME-D. This clone  
5 was isolated and the sequence was analyzed. Although this clone was incomplete in the 3' end of the translated region, ME-7 and ME-D overlapped and together covered the complete sequence of mouse ALK-3. The predicted amino-acid sequence of mouse ALK-3 is very similar to the human  
10 sequence; only 8 amino-acid residues differ (98% identity; see SEQ ID No. 14) and the calculated molecular mass of the primary translated product without the putative signal sequence is 57,447 Da.

Of the clones obtained from the initial library  
15 screening with PCR product 11.1, four clones hybridized to the probe corresponding to the conserved kinase domain of ALK-4 but not to probes from more divergent parts of ALK-1 to -4. Analysis of these clones revealed that they have an identical sequence which differs from those of ALK-1 to  
20 -5 and was termed ALK-6. The longest clone ME6 with a 2.0 kb insert was completely sequenced yielding a 1952 bp fragment consisting of an open reading frame of 1506 bp (502 amino-acids), flanked by a 5' untranslated sequence of 186 bp, and a 3' untranslated sequence of 160 bp. The  
25 nucleotide and predicted amino-acid sequences of mouse ALK-6 are shown in SEQ ID Nos. 17 and 18. No polyadenylation signal was found in the 3' untranslated region of ME6, indicating that the cDNA was internally primed in the 3' end. Only one ATG codon was found in the 5' part of the  
30 open reading frame, which fulfils the rules for translation initiation (Kozak, supra), and was preceded by an in-frame stop codon at nucleotides 163-165. However, a typical hydrophobic leader sequence was not observed at the N terminus of the translated region. Since there is no ATG  
35 codon and putative hydrophobic leader sequence, this ATG codon is likely to be used as a translation initiation site. The calculated molecular mass of the primary

translated product with the putative signal sequence is 55,576 Da.

5 Mouse ALK-1 (clone AM6 with 1.9 kb insert) was obtained from the mouse placenta  $\lambda$ ZAPII cDNA library using human ALK-1 cDNA as a probe (see SEQ ID No. 11). Mouse ALK-4 (clone 8a1 with 2.3kb insert) was also obtained from this library using human ALK-4 cDNA library as a probe (SEQ ID No. 15).

10 To summarise, clones HP22, HP57, ONF1, ONF3, ONF4 and HP29 encode the same gene, ALK-1. Clone AM6 encodes mouse ALK-1. HP53, HP64 and HP84 encode the same gene, ALK-2. ONF5, ONF2 and ON11 encode the same gene ALK-3. ME-7 and ME-D encode the mouse counterpart of human ALK-3. 11H8 encodes a different gene ALK-4, whilst 8a1 encodes the mouse equivalent. EMBLA encodes ALK-5, and ME-6 encodes ALK-6.

20 The sequence alignment between the 6 ALK genes and T $\beta$ R-II, mActR-II and ActR-IIB is shown in Figure 3. These molecules have a similar domain structure; an N-terminal predicted hydrophobic signal sequence (von Heijne (1986) Nucl. Acids Res. 14: 4683-4690) is followed by a relatively small extracellular cysteine-rich ligand binding domain, a single hydrophobic transmembrane region (Kyte & Doolittle (1982) J. Mol. Biol. 157, 105-132) and a C-terminal intracellular portion, which consists almost entirely of a kinase domain (Figures 3 and 4).

30 The extracellular domains of these receptors have cysteine-rich regions, but they show little sequence similarity; for example, less than 20% sequence identity is found between Daf-1, ActR-II, T $\beta$ R-II and ALK-5. The ALKs appear to form a subfamily as they show higher sequence similarities (15-47% identity) in their extracellular domains. The extracellular domains of ALK-5 and ALK-4 have about 29% sequence identity. In addition, 35 ALK-3 and ALK-6 share a high degree of sequence similarity in their extracellular domains (46% identity).

The positions of many of the cysteine residues in all receptors can be aligned, suggesting that the extracellular domains may adopt a similar structural configuration. See Figure 5 for ALKs-1,-2,-3 & -5. Each of the ALKs (except ALK-6) has a potential N-linked glycosylation site, the position of which is conserved between ALK-1 and ALK-2, and between ALK-3, ALK-4 and ALK-5 (see Figure 4).

The sequence similarities in the kinase domains between daf-1, ActR-II, TBR-II and ALK-5 are approximately 40%, whereas the sequence similarity between the ALKs 1 to 6 is higher (between 59% and 90%; see Figure 6). Pairwise comparison using the Jutun-Hein sequence alignment program (Hein (1990) Meth, Enzymol., 183, 626-645), between all family members, identifies the ALKs as a separate subclass among serine/threonine kinases (Figure 7).

*See G4* The catalytic domains of kinases can be divided into 12 subdomains with stretches of conserved amino-acid residues. The key motifs are found in serine/threonine kinase receptors suggesting that they are functional kinases. The consensus sequence for the binding of ATP (Gly-X-Gly-X-X-Gly<sub>7</sub> in subdomain I followed by a Lys residue further downstream in subdomain II) is found in all the ALKs.

*Seq ID No: 24*

The kinase domains of daf-1, ActR-II, and ALKs show approximately equal sequence similarity with tyrosine and serine/threonine protein kinases. However analysis of the amino-acid sequences in subdomains VI and VIII, which are the most useful to distinguish a specificity for phosphorylation of tyrosine residues versus serine/threonine residues (Hanks et al (1988) Science 241 42-52) indicates that these kinases are serine/threonine kinases; refer to Table 2.

TABLE 2

KINASE	SUBDOMAINS	
	VIB	VIII
B Serine/threonine kinase consensus	SEQ ID No. 33 DLKPEN <sub>n</sub>	seq ID No. 43 G (T/S) XX <sub>n</sub> (Y/F) X
5 B Tyrosine kinase consensus	SEQ ID No. 39 DLAARN <sub>n</sub>	XP (I/V) (K/R) W (T/M) <sub>n</sub> seq ID No. 44
B Act R-II	SEQ ID No. 40 DIKSKN <sub>n</sub>	seq ID No. 45 GTRRYM <sub>n</sub>
B Act R-IIB	SEQ ID No. 41 DFKSKN <sub>n</sub>	seq ID No. 46 GTRRYM <sub>n</sub>
B TSR-II	SEQ ID No. 42 DLKSSN <sub>n</sub>	seq ID No. 46 GTARYM <sub>n</sub>
B ALK-I	SEQ ID No. 27 DFKSRN <sub>n</sub>	seq ID No. 29 GTKRYM <sub>n</sub>
C10 B ALK -2, -3, -4, -5, & -6	SEQ ID No. 28 DLKSKN <sub>n</sub>	seq ID No. 29 GTKRYM <sub>n</sub>

15 The sequence motifs DLKSKN (Subdomain VIB) and GTKRYM (Subdomain VIII), that are found in most of the serine/threonine kinase receptors, agree well with the consensus sequences for all protein serine/threonine kinase receptors in these regions. In addition, these receptors, except for ALK-1, do not have a tyrosine residue surrounded by acidic residues between subdomains VII and VIII, which is common for tyrosine kinases. A unique characteristic

20 of the members of the ALK serine/threonine kinase receptor family is the presence of two short inserts in the kinase domain between subdomains VIA and VIB and between subdomains X and XI. In the intracellular domain, these regions, together with the juxtamembrane part and C-

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terminal tail, are the most divergent between family members (see Figures 3 and 4). Based on the sequence similarity with the type II receptors for TGF- $\beta$  and activin, the C termini of the kinase domains of ALKs -1 to -6 are set at Ser-495, Ser-501, Ser-527, Gln-500, Gln-498 and Ser-497, respectively.

#### mRNA Expression

The distribution of ALK-1, -2, -3, -4 was determined by Northern blot analysis. A Northern blot filter with mRNAs from different human tissues was obtained from Clontech (Palo Alto, C.A.). The filters were hybridized with  $^{32}\text{P}$ -labelled probes at 42°C overnight in 50% formaldehyde, 5 x standard saline citrate (SSC; 1xSSC is 50mM sodium citrate, pH 7.0, 150 mM NaCl), 0.1% SDS, 50 mM sodium phosphate, 5 x Denhardt's solution and 0.1 mg/ml salmon sperm DNA. In order to minimize cross-hybridization, probes were used that did not encode part of the kinase domains, but corresponded to the highly diverged sequences of either 5' untranslated and ligand-binding regions (probes for ALK-1, -2 and -3) or 3' untranslated sequences (probe for ALK-4). The probes were labelled by random priming using the Multiprime (or Megaprime) DNA labelling system and [ $\alpha$ - $^{32}\text{P}$ ] dCTP (Feinberg & Vogelstein (1983) Anal. Biochem. 132: 6-13). Unincorporated label was removed by Sephadex G-25 chromatography. Filters were washed at 65°C, twice for 30 minutes in 2.5 x SSC, 0.1% SDS and twice for 30 minutes in 0.3 x SSC, 0.1% SDS before being exposed to X-ray film. Stripping of blots was performed by incubation at 90-100°C in water for 20 minutes.

Our further analysis suggest ALK-1 is endothelial cell specific.

The ALK-5 mRNA size and distribution were determined by Northern blot analysis as above. An EcoR1 fragment of 980bp of the full length ALK-5 cDNA clone, corresponding to the C-terminal part of the kinase domain and 3' untranslated region (nucleotides 1259-2232 in SEQ ID No. 9)

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was used as a probe. The filter was washed twice in 0.5 x SSC, 0.1% SDS at 55°C for 15 minutes.

Using the probe for ALK-1, two transcripts of 2.2 and 4.9kb were detected. The ALK-1 expression level varied strongly between different tissues, high in placenta and lung, moderate in heart, muscle and kidney, and low (to not detectable) in brain, liver and pancreas. The relative ratios between the two transcripts were similar in most tissues; in kidney, however, there was relatively more of the 4.9 kb transcript. By reprobng the blot with a probe for ALK-2, one transcript of 4.0 kb was detected with a ubiquitous expression pattern. Expression was detected in every tissue investigated and was highest in placenta and skeletal muscle. Subsequently the blot was reprobred for ALK-3. One major transcript of 4.4 kb and a minor transcript of 7.9 kb were detected. Expression was high in skeletal muscle, in which also an additional minor transcript of 10 kb was observed. Moderate levels of ALK-3 mRNA were detected in heart, placenta, kidney and pancreas, and low (to not detectable) expression was found in brain, lung and liver. The relative ratios between the different transcripts were similar in the tested tissues, the 4.4 kb transcript being the predominant one, with the exception for brain where both transcripts were expressed at a similar level. Probing the blot with ALK-4 indicated the presence of a transcript with the estimated size of 5.2 kb and revealed an ubiquitous expression pattern. The results of Northern blot analysis using the probe for ALK-5 showed that a 5.5 kb transcript is expressed in all human tissues tested, being most abundant in placenta and least abundant in brain and heart.

The distribution of mRNA for mouse ALK-3 and -6 in various mouse tissues was also determined by Northern blot analysis. A multiple mouse tissue blot was obtained from Clontech, Palo Alto, California, U.S.A. The filter was hybridized as described above with probes for mouse ALK-3 and ALK-6. The EcoRI-PstI restriction fragment,

corresponding to nucleotides 79-1100 of ALK-3, and the SacI-HpaI fragment, corresponding to nucleotides 57-720 of ALK-6, were used as probes. The filter was washed at 65°C twice for 30 minutes in 2.5 x SSC, 0.1% SDS and twice for 30 minutes with 0.3 x SSC, 0.1% SDS and then subjected to autoradiography.

Using the probe for mouse ALK-3, a 1.1 kb transcript was found only in spleen. By reprobing the blot with the ALK-6 specific probe, a transcript of 7.2 kb was found in brain and a weak signal was also seen in lung. No other signal was seen in the other tissues tested, i.e. heart, liver, skeletal muscle, kidney and testis.

All detected transcript sizes were different, and thus no cross-reaction between mRNAs for the different ALKs was observed when the specific probes were used. This suggests that the multiple transcripts of ALK-1 and ALK-3 are coded from the same gene. The mechanism for generation of the different transcripts is unknown at present; they may be formed by alternative mRNA splicing, differential polyadenylation, use of different promoters, or by a combination of these events. Differences in mRNA splicing in the regions coding for the extracellular domains may lead to the synthesis of receptors with different affinities for ligands, as was shown for mActR-IIB (Attisano et al (1992) Cell 68, 97-108) or to the production of soluble binding protein.

The above experiments describe the isolation of nucleic acid sequences coding for new family of human receptor kinases. The cDNA for ALK-5 was then used to determine the encoded protein size and binding properties.

Properties of the ALKs cDNA Encoded Proteins

To study the properties of the proteins encoded by the different ALK cDNAs, the cDNA for each ALK was subcloned into a eukaryotic expression vector and transfected into various cell types and then subjected to immunoprecipitation using a rabbit antiserum raised against a synthetic peptide corresponding to part of the

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intracellular juxtamembrane region. This region is divergent in sequence between the various serine/threonine kinase receptors. The following amino-acid residues were used:

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ALK-1	145-166
ALK-2	151-172
ALK-3	181-202
ALK-4	153-171
10 ALK-5	158-179
ALK-6	151-168

The rabbit antiserum against ALK-5 was designated VPN.

The peptides were synthesized with an Applied Biosystems 430A Peptide Synthesizer using t-butoxycarbonyl chemistry and purified by reversed-phase high performance liquid chromatography. The peptides were coupled to keyhole limpet haemocyanin (Calbiochem-Behring) using glutaraldehyde, as described by Guillick et al (1985) EMBO J. 4, 2869-2877. The coupled peptides were mixed with Freund's adjuvant and used to immunize rabbits.

#### Transient transfection of the ALK-5 cDNA

COS-1 cells (American Type Culture Collection) and the R mutant of Mv1Lu cells (for references, see below) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 50  $\mu$ g/ml streptomycin in 5% CO<sub>2</sub> atmosphere at 37°C. The ALK-5 cDNA (nucleotides (-76) - 2232), which includes the complete coding region, was cloned in the pSV7d vector (Truett et al, (1985) DNA 4, 333-349), and used for transfection. Transfection into COS-1 cells was performed by the calcium phosphate precipitation method (Wigler et al (1979) Cell 16, 777-785). Briefly, cells were seeded into 6-well cell culture plates at a density of  $5 \times 10^5$  cells/well, and transfected the following day with 10  $\mu$ g of recombinant plasmid. After overnight incubation, cells were washed three times with a buffer containing 25 mM

Tris-HCl, pH 7.4, 138 mM NaCl, 5 mM KCl, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub> and 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, and then incubated with Dulbecco's modified Eagle's medium containing FBS and antibiotics. Two days after transfection, the cells were

5 metabolically labelled by incubating the cells for 6 hours in methionine and cysteine-free MCDB 104 medium with 150  $\mu$ Ci/ml of [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine (in vivo labelling mix; Amersham). After labelling, the cells were washed with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, and then

10 solubilized with a buffer containing 20mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1.5% Trasylol (Bayer) and 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma). After 15 minutes on ice, the cell lysates were pelleted by centrifugation, and the supernatants were

15 then incubated with 7  $\mu$ l of preimmune serum for 1.5 hours at 4°C. Samples were then given 50  $\mu$ l of protein A-Sepharose (Pharmacia-LKB) slurry (50% packed beads in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.2% Triton X100) and incubated for 45 minutes at 4°C. The beads were spun down

20 by centrifugation, and the supernatants (1 ml) were then incubated with either 7  $\mu$ l of preimmune serum or the VPN antiserum for 1.5 hours at 4°C. For blocking, 10  $\mu$ g of peptide was added together with the antiserum. Immune complexes were then given 50  $\mu$ l of protein A-Sepharose

25 (Pharmacia - LKB) slurry (50% packed beads in 150 mM NaCl, 20mM Tris-HCl, pH 7.4, 0.2% Triton X-100) and incubated for 45 minutes at 4°C. The beads were spun down and washed four times with a washing buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.2%

30 SDS), followed by one wash in distilled water. The immune complexes were eluted by boiling for 5 minutes in the SDS-sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) in the presence of 10 mM DTT, and analyzed by SDS-gel electrophoresis using 7-15%

35 polyacrylamide gels (Blobel and Dobberstein, (1975) J.Cell Biol. 67, 835-851). Gels were fixed, incubated with Amplify (Amersham) for 20 minutes, and subjected to

fluorography. A component of 53Da was seen. This component was not seen when preimmune serum was used, or when 10  $\mu$ g blocking peptide was added together with the antiserum. Moreover, it was not detectable in samples derived from untransfected COS-1 cells using either preimmune serum or the antiserum.

#### Digestion with Endoglycosidase F

Samples immunoprecipitated with the VPN antisera obtained as described above were incubated with 0.5 U of endoglycosidase F (Boehringer Mannheim Biochemica) in a buffer containing 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% Triton X-100, 0.1% SDS and 1%  $\beta$ -mercaptoethanol at 37°C for 24 hours. Samples were eluted by boiling for 5 minutes in the SDS-sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis as described above. Hydrolysis of N-linked carbohydrates by endoglycosidase F shifted the 53 kDa band to 51 kDa. The extracellular domain of ALK-5 contains one potential acceptor site for N-glycosylation and the size of the deglycosylated protein is close to the predicted size of the core protein.

#### Establishment of PAE Cell Lines Expressing ALK-5

In order to investigate whether the ALK-5 cDNA encodes a receptor for TGF- $\beta$ , porcine aortic endothelial (PAE) cells were transfected with an expression vector containing the ALK-5 cDNA, and analyzed for the binding of  $^{125}$ I-TGF- $\beta$ 1.

PAE cells were cultured in Ham's F-12 medium supplemented with 10% FBS and antibiotics (Miyazono *et al.*, (1988) J. Biol. Chem. 263, 6407-6415). The ALK-5 cDNA was cloned into the cytomegalovirus (CMV)-based expression vector pcDNA I/NEO (Invitrogen), and transfected into PAE cells by electroporation. After 48 hours, selection was initiated by adding Geneticin (G418 sulphate; Gibco - BRL) to the culture medium at a final concentration of 0.5 mg/ml (Westermarck *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87, 128-132). Several clones were obtained, and after analysis by immunoprecipitation using the VPN antiserum, one clone denoted PAE/T $\beta$ R-1 was chosen and further analyzed.

Iodination of TGF- $\beta$ 1, Binding and Affinity Crosslinking

Recombinant human TGF- $\beta$ 1 was iodinated using the chloramine T method according to Frolik *et al.*, (1984) J. Biol. Chem. 259, 10995-11000. Cross-linking experiments were performed as previously described (Ichijo *et al.*, (1990) Exp. Cell Res. 187, 263-269). Briefly, cells in 6-well plates were washed with binding buffer (phosphate-buffered saline containing 0.9 mM CaCl<sub>2</sub>, 0.49 mM MgCl<sub>2</sub> and 1 mg/ml bovine serum albumin (BSA)), and incubated on ice in the same buffer with <sup>125</sup>I-TGF- $\beta$ 1 in the presence or absence of excess unlabelled TGF- $\beta$ 1 for 3 hours. Cells were washed and cross-linking was done in the binding buffer without BSA together with 0.28 mM disuccinimidyl suberate (DSS; Pierce Chemical Co.) for 15 minutes on ice. The cells were harvested by the addition of 1 ml of detachment buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 0.3 mM PMSF). The cells were pelleted by centrifugation, then resuspended in 50  $\mu$ l of solubilization buffer (125 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.3 mM PMSF, 1% Trasylol) and incubated for 40 minutes on ice. Cells were centrifuged again and supernatants were subjected to analysis by SDS-gel electrophoresis using 4-15% polyacrylamide gels, followed by autoradiography. <sup>125</sup>I-TGF- $\beta$ 1 formed a 70 kDa cross-linked complex in the transfected PAE cells (PAE/T $\beta$ R-I cells). The size of this complex was very similar to that of the TGF- $\beta$  type I receptor complex observed at lower amounts in the untransfected cells. A concomitant increase of 94 kDa TGF- $\beta$  type II receptor complex could also be observed in the PAE/T $\beta$ R-I cells. Components of 150-190 kDa, which may represent crosslinked complexes between the type I and type II receptors, were also observed in the PAE/T $\beta$ R-I cells.

In order to determine whether the cross-linked 70 kDa complex contained the protein encoded by the ALK-5 cDNA, the affinity cross-linking was followed by immunoprecipitation using the VPN antiserum. For this, cells in 25 cm<sup>2</sup> flasks were used. The supernatants obtained after cross-linking were incubated with 7  $\mu$ l of preimmune serum or VPN antiserum in the presence or absence of 10  $\mu$ g of peptide for 1.5h at 4°C. Immune complexes were then added to 50  $\mu$ l of protein A-Sepharose slurry and incubated for 45 minutes at 4°C. The protein A-Sepharose beads were washed four times with the washing buffer, once with distilled water, and the samples were analyzed by SDS-gel electrophoresis using 4-15% polyacrylamide gradient gels and autoradiography. A 70 kDa cross-linked complex was precipitated by the VPN antiserum in PAE/T $\beta$ R-1 cells, and a weaker band of the same size was also seen in the untransfected cells, indicating that the untransfected PAE cells contained a low amount of endogenous ALK-5. The 70 kDa complex was not observed when preimmune serum was used, or when immune serum was blocked by 10  $\mu$ g of peptide. Moreover, a coprecipitated 94 kDa component could also be observed in the PAE/T $\beta$ R-I cells. The latter component is likely to represent a TGF- $\beta$  type II receptor complex, since an antiserum, termed DRL, which was raised against a synthetic peptide from the C-terminal part of the TGF- $\beta$  type II receptor, precipitated a 94 kDa TGF- $\beta$  type II receptor complex, as well as a 70 kDa type I receptor complex from PAE/T $\beta$ R-I cells.

The carbohydrate contents of ALK-5 and the TGF- $\beta$  type II receptor were characterized by deglycosylation using endoglycosidase F as described above and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The ALK-5 cross-linked complex shifted from 70 kDa to 66 kDa, whereas that of the type II receptor shifted from 94 kDa to 82 kDa. The observed larger shift of the type II receptor band compared with that of the ALK-5 band is consistent with the deglycosylation data of the type I and

type II receptors on rat liver cells reported previously (Cheifetz et al (1988) J. Biol. Chem. 263, 16984-16991), and fits well with the fact that the porcine TGF- $\beta$  type II receptor has two N-glycosylation sites (Lin et al (1992) Cell 68, 775-785), whereas ALK-5 has only one (see SEQ ID No. 9).

Binding of TGF- $\beta$ 1 to the type I receptor is known to be abolished by transient treatment of the cells with dithiothreitol (DTT) (Cheifetz and Massague (1991) J. Biol. Chem. 266, 20767-20772; Wrana et al (1992) Cell 71, 1003-1014). When analyzed by affinity cross-linking, binding of  $^{125}$ I-TGF- $\beta$ 1 to ALK-5, but not to the type II receptor, was completely abolished by DTT treatment of PAE/T $\beta$ R-1 cells. Affinity cross-linking followed by immunoprecipitation by the VPN antiserum showed that neither the ALK-5 nor the type II receptor complexes was precipitated after DTT treatment, indicating that the VPN antiserum reacts only with ALK-5. The data show that the VPN antiserum recognizes a TGF- $\beta$  type I receptor, and that the type I and type II receptors form a heteromeric complex.

#### $^{125}$ I-TGF- $\beta$ 1 Binding & Affinity Crosslinking of Transfected COS Cells

Transient expression plasmids of ALKs -1 to -6 and T $\beta$ R-II were generated by subcloning into the pSV7d expression vector or into the pcDNA I expression vector (Invitrogen). Transient transfection of COS-1 cells and iodination of TGF- $\beta$ 1 were carried out as described above. Crosslinking and immunoprecipitation were performed as described for PAE cells above.

Transfection of cDNAs for ALKs into COS-1 cells did not show any appreciable binding of  $^{125}$ I-TGF $\beta$ 1, consistent with the observation that type I receptors do not bind TGF- $\beta$  in the absence of type II receptors. When the T $\beta$ R-II cDNA was co-transfected with cDNAs for the different ALKs, type I receptor-like complexes were seen, at different levels, in each case. COS-1 cells transfected with T $\beta$ R-II

and ALK cDNAs were analyzed by affinity crosslinking followed by immunoprecipitation using the DRL antisera or specific antisera against ALKs. Each one of the ALKs bound  $^{125}\text{I}$ -TGF- $\beta$ 1 and was coimmunoprecipitated with the T $\beta$ R-II complex using the DRL antiserum. Comparison of the efficiency of the different ALKs to form heteromeric complexes with T $\beta$ R-II, revealed that ALK-5 formed such complexes more efficiently than the other ALKs. The size of the crosslinked complex was larger for ALK-3 than for other ALKs, consistent with its slightly larger size.

#### Expression of the ALK Protein in Different Cell Types

Two different approaches were used to elucidate which ALK's are physiological type I receptors for TGF- $\beta$ .

Firstly, several cell lines were tested for the expression of the ALK proteins by cross-linking followed by immunoprecipitation using the specific antisera against ALKs and the TGF- $\beta$  type II receptor. The mink lung epithelial cell line, Mv1Lu, is widely used to provide target cells for TGF- $\beta$  action and is well characterized regarding TGF- $\beta$  receptors (Laiho et al (1990) J. Biol. Chem. 265, 18518-18524; Laiho et al (1991) J. Biol. Chem. 266, 9108-9112). Only the VPN antiserum efficiently precipitated both type I and type II TGF- $\beta$  receptors in the wild type Mv1Lu cells. The DRL antiserum also precipitated components with the same size as those precipitated by the VPN antiserum. A mutant cell line (R mutant) which lacks the TGF- $\beta$  type I receptor and does not respond to TGF- $\beta$  (Laiho et al, supra) was also investigated by cross-linking followed by immunoprecipitation. Consistent with the results obtained by Laiho et al (1990), supra the type III and type II TGF- $\beta$  receptor complexes, but not the type I receptor complex, were observed by affinity crosslinking. Crosslinking followed by immunoprecipitation using the DRL antiserum revealed only the type II receptor complex, whereas neither the type I nor type II receptor complexes was seen using the VPN antiserum. When the cells were metabolically labelled and subjected to immunoprecipitation

using the VPN antiserum, the 53 kDa ALK-5 protein was precipitated in both the wild-type and R mutant Mv1Lu cells. These results suggest that the type I receptor expressed in the R mutant is ALK-5, which has lost the affinity for binding to TGF- $\beta$  after mutation.

The type I and type II TGF- $\beta$  receptor complexes could be precipitated by the VPN and DRL antisera in other cell lines, including human foreskin fibroblasts (AG1518), human lung adenocarcinoma cells (A549), and human oral squamous cell carcinoma cells (HSC-2). Affinity cross-linking studies revealed multiple TGF- $\beta$  type I receptor-like complexes of 70-77 kDa in these cells. These components were less efficiently competed by excess unlabelled TGF- $\beta$ 1 in HSC-2 cells. Moreover, the type II receptor complex was low or not detectable in A549 and HSC-2 cells. Cross-linking followed by immunoprecipitation revealed that the VPN antiserum precipitated only the 70 kDa complex among the 70-77 kDa components. The DRL antiserum precipitated the 94 kDa type II receptor complex as well as the 70 kDa type I receptor complex in these cells, but not the putative type I receptor complexes of slightly larger sizes. These results suggest that multiple type I TGF- $\beta$  receptors may exist and that the 70 kDa complex containing ALK-5 forms a heteromeric complex with the TGF- $\beta$  type II receptor cloned by Lin *et al* (1992) Cell 68, 775-785, more efficiently than the other species. In rat pheochromocytoma cells (PC12) which have been reported to have no TGF- $\beta$  receptor complexes by affinity cross-linking (Massagué *et al* (1990) Ann. N.Y. Acad. Sci. 593, 59-72), neither VPN nor DRL antisera precipitated the TGF- $\beta$  receptor complexes. The antisera against ALKs -1 to -4 and ALK6 did not efficiently immunoprecipitate the crosslinked receptor complexes in porcine aortic endothelial (PAE) cells or human foreskin fibroblasts.

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Next, it was investigated whether ALKs could restore responsiveness to TGF- $\beta$  in the R mutant of Mv1Lu cells, which lack the ligand-binding ability of the TGF- $\beta$  type I receptor but have intact type II receptor. Wild-type Mv1Lu cells and mutant cells were transfected with ALK cDNA and were then assayed for the production of plasminogen activator inhibitor-1 (PAI-1) which is produced as a result of TGF- $\beta$  receptor activation as described previously by Laiho *et al* (1991) Mol. Cell Biol. 11, 972-978. Briefly, cells were added with or without 10 ng/ml of TGF- $\beta$ 1 for 2 hours in serum-free MCDB 104 without methionine. Thereafter, cultures were labelled with [ $^{35}$ S] methionine (40  $\mu$ Ci/ml) for 2 hours. The cells were removed by washing on ice once in PBS, twice in 10 mM Tris-HCl (pH 8.0), 0.5% sodium deoxycholate, 1 mM PMSF, twice in 2 mM Tris-HCl (pH 8.0), and once in PBS. Extracellular matrix proteins were extracted by scraping cells into the SDS-sample buffer containing DTT, and analyzed by SDS-gel electrophoresis followed by fluorography using Amplify. PAI-1 can be identified as a characteristic 45kDa band (Laiho *et al* (1991) Mol. Cell Biol. 11, 972-978). Wild-type Mv1Lu cells responded to TGF- $\beta$  and produced PAI-1, whereas the R mutant clone did not, even after stimulation by TGF- $\beta$ 1. Transient transfection of the ALK-5 cDNA into the R mutant clone led to the production of PAI-1 in response to the stimulation by TGF- $\beta$ 1, indicating that the ALK-5 cDNA encodes a functional TGF- $\beta$  type I receptor. In contrast, the R mutant cells that were transfected with other ALKs did not produce PAI-1 upon the addition of TGF- $\beta$ 1.

Using similar approaches as those described above for the identification of TGF- $\beta$ -binding ALKs, the ability of ALKs to bind activin in the presence of ActRII was examined. COS-1 cells were co-transfected as described above. Recombinant human activin A was iodinated using the chloramine T method (Mathews and Vale (1991) Cell 65, 973-982). Transfected COS-1 cells were analysed for binding and crosslinking of  $^{125}$ I-activin A in the presence or

absence of excess unlabelled activin A. The crosslinked complexes were subjected to immunoprecipitation using DRL antisera or specific ALK antisera.

5 All ALKs appear to bind activin A in the presence of Act R-II. This is more clearly demonstrated by affinity cross-linking followed by immunoprecipitation. ALK-2 and ALK-4 bound  $^{125}$ I-activin A and were coimmunoprecipitated with ActR-II. Other ALKs also bound  $^{125}$ I-activin A but with a lower efficiency compared to ALK-2 and ALK-4.

10 In order to investigate whether ALKs are physiological activin type I receptors, activin responsive cells were examined for the expression of endogenous activin type I receptors. Mv1Lu cells, as well as the R mutant, express both type I and type II receptors for activin, and the R mutant cells produce PAI-1 upon the addition of activin A. 15 Mv1Lu cells were labeled with  $^{125}$ I-activin A, cross-linked and immunoprecipitated by the antisera against ActR-II or ALKs as described above.

The type I and type II receptor complexes in Mv1Lu 20 cells were immunoprecipitated only by the antisera against ALK-2, ALK-4 and ActR-II. Similar results were obtained using the R mutant cells. PAE cells do not bind activin because of the lack of type II receptors for activin, and so cells were transfected with a chimeric receptor, to 25 enable them to bind activin, as described herein. A plasmid (chim A) containing the extracellular domain and C-terminal tail of Act R-II (amino-acids -19 to 116 and 465 to 494, respectively (Mathews and Vale (1991) Cell, 65, 973-982)) and the kinase domain of T $\beta$ R-II (amino-acids 160- 30 543) (Lin et al (1992) Cell, 68, 775-785) was constructed and transfected into pcDNA/neo (Invitrogen). PAE cells were stably transfected with the chim A plasmid by electroporation, and cells expressing the chim A protein were established as described previously. PAE/Chim A cells 35 were then subjected to  $^{125}$ I-activin A labelling crosslinking and immunoprecipitation as described above.

Similar to Mv1Lu cells, activin type I receptor complexes in PAE/Chim A cells were immunoprecipitated by the ALK-2 and ALK-4 antisera. These results show that both ALK-2 and ALK-4 serve as high affinity type I receptors for  
 5 activin A in these cells.

ALK-1, ALK-3 and ALK-6 bind TGF- $\beta$ 1 and activin A in the presence of their respective type II receptors, but the functional consequences of the binding of the ligands remains to be elucidated.

10 The experiments described supra suggested further experiments. Specifically, it is known that TGF- $\beta$  family members acts as ligands in connection with specific type I and type II receptors, with resulting complexes interacting with members of the Smad family. See Heldin  
 15 et al., Nature 390: 465-471 (1997), incorporated by reference. The Smad molecules are homologs of molecules found in Drosophila ("Mad"), and C. elegans (Sma), hence, the acronym "Smad". These are involved in signal transduction pathways downstream of serine/threonine kinase  
 20 receptors. See Massagué et al., Trends Cell Biol. 2: 187-192 (1997). The different members of the family have different signaling roles. Smad1, for example, as well as Smad 2 and 3, and perhaps Smad 5, became phosphorylated via specific type 1 serine/threonine kinase receptors, and act  
 25 in pathway restricted fashion. For example, Xenopus Mad1 induces ventral mesoderm, in the presence of BMP. The human Smad1 has been shown to have ventralizing activity. See Liu et al., Nature 381: 620-623 (1996); Kretzschmer et al., Genes Dev 11: 984-995 (1997). There is also some  
 30 evidence that TGF- $\beta$  phosphorylates Smad1. See Lechleider et al., J. Biol. Chem. 271: 17617-17620 (1996); Yingling et al., Proc. Natl. Acad. Sci. USA 93: 8940-8944 (1996). Given what was known regarding this complex signaling pathway, the role of ALK-1 was studied.

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COS-7 cells, which do not express ALK-1, were transfected with cDNA encoding tagged ALK-1. The tag was hemagglutinin (hereafter "HA"), and a commercially available lipid containing transfecting agent was used.

5 In parallel experiments, porcine aortic endothelial (PAE) cells were also used, because these cells express TGF $\beta$  type II receptors, and ALK-5, but not ALK-1. Hence, PAE cells were either transfected, or not. Transfection protocols are given, supra.

10 The cells were then contacted with  $^{125}$ I labelled TGF- $\beta$ 1, and were then contacted with ALK-1 specific antisera, to ascertain whether cross linking had occurred. See the experiments, supra, as well as ten Dijke et al., Science 264: 101-104 (1994), incorporated by reference. Antisera  
15 to ALK-5 were also used.

The results indicated that the ALK-1 antiserum immunoprecipitated complexes of the appropriate size from the transfected COS-7 and PAE cells, but not those which were not transfected, thereby establishing that ALK-1 is  
20 a receptor for TGF- $\beta$ .

This was confirmed in experiments on human umbilical vein endothelial cells (HUVEC). These cells are known to express ALK-1 endogenously, as well as ALK-5. The ALK-5 antiserum and the ALK-1 antiserum both immunoprecipitated  
25 type I and type II receptor cross linked complexes. The ALK-1 antiserum immunoprecipitated band migrated slightly more slowly than the band immunoprecipitated by the ALK-5 antiserum (see, e.g., Fig. 8). This is in agreement with the difference in size of ALK-1 and ALK-5, and it indicates  
30 that both ALK-1 and ALK-5 bind TGF- $\beta$  in HUVECS.

Further, it shows that ALK-1 acts as a co-called "type I" TGF- $\beta$  receptor in an endogenous, physiological setting.

Once it was determined that TGF- $\beta$  and ALK-1 interact, studies were carried out to determine whether or not  
35 activation of ALK-1 resulted in phosphorylation of Smads. To test this, COS-7 cells were transfected in the same manner described supra with either Flag tagged Smad1, Flag

tagged Smad2 or Flag tagged Smad-5 together with either a constitutively active form of ALK-1, or a constitutively active form of ALK-5. Specifically, the variant of ALK-1 is Q201D, and that of ALK-5 is T204D. Constitutively active ALK-1 was used to avoid the need for an additional transfection step. To elaborate, it is known that for the TGF- $\beta$  pathway to function adequately, a complex of two, type I receptors, and two, type II receptors must interact, so as to activate the receptors. Constitutively active receptors, such as what was used herein, do not require the presence of the type II receptor to function. See Wieser et al., EMBO J 14: 2199-2208 (1995). In order to determine if the resulting transfected cells produced phosphorylated Smads, Smads were determined using a Flag specific antibody, which precipitated them, and phosphorylation was determined using the antiphosphoserine antibody of Nishimura et al., J. Biol. Chem. 273: 1872-1879 (1998). It was determined, when the data were analyzed, that Smad1 and Smad-5 (an intracellular signalling molecule which is structurally highly similar to Smad1) were phosphorylated following interaction with activated ALK-1, but not following interaction of TGF- $\beta$  and ALK-5. Conversely, the interaction of TGF- $\beta$  and ALK-5 led to phosphorylation of Smad 2, but not Smad 1. This supports a conclusion that ALK-1 transduces signal in a manner similar to BMPs.

Figure 8 depicts the phosphorylation of Smad-5 following interaction with ALK-1 but not ALK-5. Phosphorylation of both Smad-5 and Smad1 has been shown for BMP type I receptors suggesting ALK-1 is functionally very similar to ALK3 (BMPRI-A) and (ALK6 BMPRI-B).

Additional experiments were then carried out to study the interaction of ALK-1 with Smad-1. Specifically, COS-7 cells were transfected with cDNA which encoded the wild type form of the TGF $\beta$  type II receptor (TBR-II), a kinase inactive form of ALK-1, and Flag tagged Smad-1. Kinase inactive ALK-1 was used, because the interaction of Smad-1 and receptors is known to be transient, as once Smads are

phosphorylated they dissociate from the type I receptor. See Marcias-Silva et al., Cell 87: 1215-1224 (1996); Nakao et al., EMBO J 16: 5353-5362 (1997). Affinity cross-linking, using  $^{125}\text{I}$ -TGF- $\beta$ 1, and immunoprecipitation with  
5 Flag antibody was carried out, as discussed supra. The expression of ALK-1 was determined using anti-HA antibody, since the vector used to express ALK-1 effectively tagged it with HA.

10 The immunoprecipitating of Smad1 resulted in coprecipitation of a cross linked TBR-II/ALK-1 complex, suggesting a direct association of Smad1 with ALK-1.

These examples show that one can identify molecules which inhibit, or enhance expression of a gene whose expression is regulated by phosphorylated Smad1. To  
15 elaborate, as ALK-1 has been identified as a key constituent of the pathway by which Smad1 is phosphorylated, one can contact cells which express both Smad1 and ALK-1 with a substance of interest, and then determine if the Smad1 becomes phosphorylated. The cells  
20 can be those which inherently express both ALK-1 and Smad1, or which have been transformed or transfected with DNA encoding one or both of these. One can determine the phosphorylation via, e.g., the use of anti phosphorylated serine antibodies, as discussed supra. In an especially  
25 preferred embodiment, the assay can be carried out using TGF- $\beta$ , as a competing agent. The TGF- $\beta$ , as has been shown, does bind to ALK-1, leading to phosphorylation of Smad1. Hence, by determining a value with TGF- $\beta$  alone, one can then compare a value determined with amounts of the  
30 substance to be tested, in the presence of TGF- $\beta$ . Changes in phosphorylation levels can thus be attributed to the test substance.

In this type of system, it must be kept in mind that both type I receptors and type II receptors must be  
35 present; however, as indicated, supra, one can eliminate the requirement for a type II receptor by utilizing a constitutively active form of ALK-1, such as the form

described supra. Additional approaches to inhibiting this system will be clear to the skilled artisan. For example, since it is known that there is interaction between Smad1 and the ALK-1 receptor, one can test for inhibition via the use of small molecules which inhibit the receptor/Smad interaction. Heldin et al., supra, mention Smad6 and Smad7 as Smad1 inhibitors, albeit in the context of a different system. Hence one can test for inhibition, or inhibit the interaction, via adding a molecule to be tested or for actual inhibition to a cell, wherein the molecule is internalized by the cell, followed by assaying for phosphorylation, via a method such as is discussed supra.

In a similar way, one can assay for inhibitors of type I/type II receptor interaction, by testing the molecule of interest in a system which includes both receptors, and then assaying for phosphorylation.

Conversely, activators or agonists can also be tested for, or utilized, following the same type of procedures.

Via using any of these systems, one can identify any gene or genes which are activated by phosphorylated Smad1. To elaborate, the art is very familiar with systems of expression analysis, such as differential display PCR, subtraction hybridization, and other systems which combine driver and testes populations of nucleic acids, whereby transcripts which are expressed or not expressed can be identified. By simply using an activator/inhibitor of the system disclosed herein, on a first sample, and a second sample where none is used, one can then carry out analysis of transcript, thereby determining the transcripts of interest.

Also a part of the invention is the regulation of a phosphorylation of Smad-1 or Smad-5, with inhibitors, such as antibodies against the extracellular domain of ALK-1 or TGF- $\beta$ , or enhancers, such as TGF- $\beta$  itself, or those portions of the TGF- $\beta$  molecule which are necessary for binding. Indeed, by appropriate truncation, one can also

determine what portions of ALK-1 are required for phosphorylation of Smad1 or Smad-5 to take place.

The invention has been described by way of example only, without restriction of its scope. The invention is defined by the subject matter herein, including the claims  
5 that follow the immediately following full Sequence Listings.

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